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Original Article

Effects of exogenous hydrogen sulfide on sepsis-induced oxidative stress in myocardial mitochondrial

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Abstract: Objectives: The study aimed to investigate the effect of exogenous hydrogen sulfide on oxidative stress in myocardial mitochondria during CLP-induced sepsis. Methods: The CLP-sepsis model was built and treated by different concentrations of NaHS. The degree of myocardial mitochondria damage was evaluated by the degree of swelling and the ATP level. The oxidative stress level was measured by the ROS and MDA levels. The mRNA and protein levels of HO-1 and MnSOD of myocardial mitochondria were analyzed. The relationship was analyzed between the ATP, ROS, and MDA level, and the mRNA and protein level of HO-1 and MnSOD after NaHS treatment. Results: In the sepsis group, the degree of myocardial mitochondria swelling was aggravated, the ATP level was decreased, the ROS ratio, the MDA level, the mRNA and protein level of HO-1 and MnSOD were increased. After NaHS treatment, the degree of myocardial mitochondria swelling was alleviated, the ATP level was increased, the ROS ratio and the MDA level were reduced, and the mRNA and protein level of HO-1 and MnSOD were increased further concentration-dependently. Moreover, in NaHS treatment group, the ATP level was positively correlated with HO-1, MnSOD mRNA level, and the ROS, MDA level were negatively correlated with HO-1 and MnSOD mRNA level. The similarcorrelation was found between the ATP, ROS, MDA level and HO-1, MnSOD protein level in 25, 50, 100 umol/L NaHS treatment group. Conclusion: Exogenous NaHS could promote the expression of HO-1 and MnSOD, lessen oxidative stress, and improve the myocardial mitochondria function in CLP-induced sepsis.

Keywords: Hydrogen sulfide, sepsis, oxidative stress, myocardial mitochondria

Introduction

Sepsis is defined as the systemic inflammatory response syndrome that occurs during infection [1]. It is a common critical illness and an important cause of mortality worldwide. Sepsis often aggravates quickly and presents with the evidence of severe sepsis, septic shock, and multiple organ dysfunction syndrome (MODS) [2]. Many studies verify that when multiple organ dysfunction is caused by sepsis, myocardial depression will appear, which can further develop into cardiac insufficiency [3]. However, the underlying pathogenesis of cardiac dysfunction caused by sepsis is still not fully clear.

In recent years, some studies have focused mainly on myocardial mitochondria. They believe that the disorder in myocardial energy metabolism is the key [4, 5]. Mitochondria are the center of energy metabolism and provide energy for cellular activities. Mitochondrial damage and dysfunction have been recognized as important molecular pathologies in sepsis [6, 7]. Increased production of cellular reactive oxygen species (ROS) of mitochondrial origin during sepsis can cause significant oxidative stress to cells and may severely inhibit adenosine triphosphate (ATP) generation, an important cause of mitochondrial damage of myocardial cell [6]. Cells defend against various oxidative stresses using a combination of physical, preventative, repair, and antioxidant defense mechanisms. An antioxidant defense system is the major protective mechanism [8]. Antioxidant defense systems are classified as enzymatic and non-enzymatic, as well as endogenous and exogenous [9]. These molecules participate in the improvement of mitochondrial function and the relief of oxidative stress. Thus, targeting oxi-

dative stress and antioxidant molecules might be an effective therapeutic method for managing mitochondrial damage in sepsis.

Hydrogen sulfide (H2S) represents the most recently identified endogenously produced gaseous messenger, which is widely present in mammalian tissues and has numerous signaling functions, thus contributing to various physiological and pathological processes [10]. H2S can inhibit ROS-mediated injury and reduce the deleterious effects of oxidative stress, as determined in a number of *in vitro* and *in vivo* studies in animal models [11, 12]. Few studies have been done on its effects in sepsis.

Sodium hydrosulfide (NaHS) is an exogenous H2S donor. Through establishing the sepsis mouse model and analyzing the function of mitochondria as well as detecting the level of oxidative stress and related antioxidant molecules, the present study is intended to clarify the influence of exogenous NaHS on redox balance after sepsis, explore the pathogenesis of sepsis, and provide a basis for research and development of new drugs.

Materials and methods

Animals and CLP model

C57BL/6 male SPF grade mice 6-8 weeks old and 20-30 g, were purchased from Beijing HFK Bioscience Co. Ltd. The mice were randomly divided into the following groups, 10 mice per group: Group A (normal control), Group B (sham-operation; cecum removal by laparotomy, replacement of cecum and suture), Group C (sepsis; cecum removal by laparotomy, cecal ligation and puncture, replacement of cecum and suture), Group D (sepsis procedure as in Group C + 25 μmol/L NaHS), Group E (sepsis procedure as in Group C + 50 μmol/L NaHS), Group F (sepsis procedure as in Group C + 100 μmol/L NaHS), and Group G (sepsis procedure as in Group C + 200 μmol/L NaHS). The mice were fed with pellet feed for mice in the cage ad libitum. The mice were acclimated for one week before the experiment. Sepsis was induced by cecal ligation puncture (CLP) procedure as previously described [13]. An intraperitoneal injection of 7% chloral hydrate solution (0.5 ml/100 g) was administered. After anesthesia, abdominal skin was prepared and a 75% alcohol disinfection was performed. With a longitudinal incision, the cecum was explored and exposed. Ligation was conducted at about 1 cm to the end of cecum, so as to achieve sepsis. After replacement of the cecum in the abdominal cavity and suture layer-by-layer, intraperitoneal injection of NaHS (Sigma) in corresponding concentration (20 ml/kg) was administered. After the operation, a hypodermic injection of normal saline (3 ml/100 g) was administered. The mice were placed in the cage until post anesthetic recovery. After 12 h, the mice were euthanized by spinal dislocation after anesthesia and the myocardial tissues were collected for subsequent analysis. All procedures and housing were in accordance with the United Kingdom Animal (Scientific Procedures) Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Myocardial mitochondria isolation and detection

Myocardial mitochondria were isolated from each group. Briefly, the heart was quickly excised and washed in buffer containing 250 mM sucrose, 10 mM Tris, 1 mM EGTA, pH 7.4 at 4°C. After changes of buffer, the myocardium samples were cut into small pieces and homogenized. The samples were centrifuged at 700 r/min for 10 min to remove debris, and the supernatant was centrifuged at 1000 r/min for 15 min, then the supernatant was removed and myocardial mitochondria were obtained. All isolated mitochondria were kept on ice and used within 3 h of isolation. Some myocardial tissues were taken from the left ventricle. Based on the protein concentration, the protein concentration of the sample was adjusted to 0.5 mg/ml by mitochondria determination liquid. The degree of myocardial mitochondria swelling was evaluated by the OD values, which were measured at 520 nm of ultraviolet pectrophotometer. The ATP level of myocardial mitochondria was detected by an ATP kit (BioVision).

Oxidative stress detection

Intracellular ROS were detected using a ROS assay kit by means of an oxidation-sensitive fluorescent probe (DCFH-DA) in a spectrofluorometer (excitation 500 nm, emission 520 nm).

Table 1. Primer of fluorescent quantitative PCR

Primer Name	Sequence (5'-3')	Amplification length (bp)
HO-1F	CGAATGAACACTCTGGAGATGAC	166
HO-1R	GCCTCTGACGAAGTGACGC	
MnSOD F	TGGACAAACCTGAGCCCTAAG	138
MnSOD R	CCAGCCTGAACCTTGGACTC	

The malondialdehyde (MDA) level was detected by the thiobarbituric acid (TBA) method.

Detection of HO-1 and MnSOD protein

The myocardial tissue was first cut into 1-mm^3 fragments, then the appropriate amount of RIPA lysate was added to achieve the homogenate. The lysed cell or tissue was centrifuged at 4°C and 12,000 g for 5 min. The supernatant was taken for SDS-PAGE and Western blot. After film scanning, the gray value of the targeted band was analyzed by UVP gel image processing system Labworks 4.6 software.

Detection of HO-1 and MnSOD mRNA

1 ml TRIzol reagent (Qiagen) was added to 50-100 mg tissue. Then the homogenate was obtained. The homogenate was centrifuged at 4°C and 12000 g for 10 min. After removal of insoluble substance, the subsequent RNA extraction steps were conducted. After total RNA was extracted, random primer reverse transcription reaction and SYBR green I fluorescent quantitative PCR (Eppendorf) were carried out. The mRNA level of HO-1 and MnSOD was detected. The primer sequence is shown in **Table 1**. The PCR condition was 94°C for 4 min, 94°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec, a total of 35 cycles. Three repeated wells were set for each sample.

Statistical analyses

Data were analyzed by the Statistical Product for Social Sciences (SPSS; version 20.0). The difference among the groups was determined by one-way analysis of variance. Bar graphs were used to describe the statistical results. The Pearson's correlation coefficient was used to describe the correlation between indicators. A p value <0.05 was regarded as of statistical significance.

Results

Swelling and function change of myocardial mitochondria

The result indicated that the difference in degree of myocardial mitochondria swelling and the ATP level between the normal control group and the sham-operation group was not statistically significant. In the sepsis group, the degree of myocardial mitochondria swelling was aggravated and the ATP level was decreased significantly. After NaHS treatment, the degree of myocardial mitochondria swelling was alleviated and the ATP level was increased significantly, in a concentration-dependent manner (**Figure 1**).

The level of oxidative stress

The ROS in the sham-operation group and the sepsis group had increased to varying degrees compared with the normal control group. NaHS affected the ROS ratio in a concentration-dependent manner (**Figure 2**). The difference of the level of MDA between the normal group and the sham-operation group was not statistically significant. It increased significantly in sepsis group. After NaHS treatment, the level of MDA was reduced significantly in a concentration-dependent manner (**Figure 3**).

Detection of HO-1 and MnSOD protein level

Western blot and gray scanning analysis indicated that there was no significant difference in the protein expression level of HO-1 and MnSOD between the normal group and the sham-operation group. The HO-1 and MnSOD level increased significantly in the sepsis group. After NaHS treatment, both increased further in a concentration-dependent manner in 25, 50, 100 umol/L NaHS group. But it decreased in 200 umol/L NaHS group compared with 100 umol/L NaHS group (**Figure 4**).

Detection of HO-1 and MnSOD mRNA level

The melting curve of fluorescent quantitative PCR indicated that primers of HO-1 and MnSOD gene had good specificity (**Figure 5**). The result of standard curve indicated that the amplification efficiency of PCR system was higher than

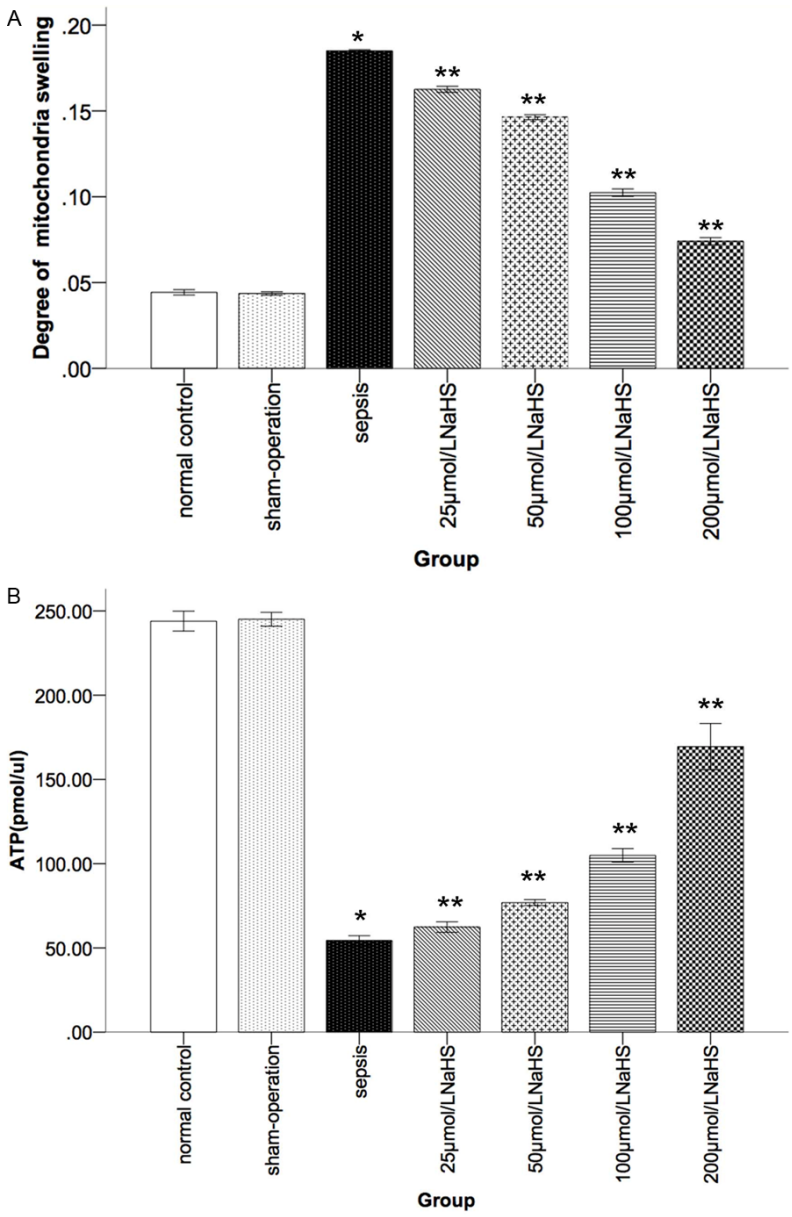

Figure 1. A: The degree of myocardial mitochondria swelling. B: The ATP level. n=10 per group. The mice were submitted to CLP procedure for sepsis induction. Meanwhile, intraperitoneal injection of NaHS in corresponding concentrations was administered. 12 h later, the mice were euthanized and myocardial tissue collected for subsequent analysis. *P<0.05 vs. sham-operation group, **P<0.05 vs. sepsis group. The error bars are indicative of the 95% confidence intervals.

95%. The relative quantification result indicated that there was no significant difference between the normal group and the sham-operation group in the mRNA level of HO-1 and MnSOD. It increased significantly in the sepsis group. After NaHS treatment, the mRNA level of HO-1 and MnSOD increased further in a dose-dependent manner (**Figure 6**).

The correlation between the level of ATP, ROS, MDA and HO-1, MnSOD level

After NaHS treatment, the ATP level was highly positively correlated with HO-1, MnSOD mRNA level (r_{HO-1}=0.839, P=0.000; r_{MnSOD}= 0.887, P=0.000), the ROS level was highly negatively correlated with HO-1, MnSOD mRNA level (r_{HO-1}=-0.756, P=0.000; r_{MnSOD}= -0.795, P=0.000), the MDA level was highly negatively correlated with HO-1, MnSOD mRNA level (r_{HO-1}=-0.904, P=0.000; r_{MnSOD}= -0.928, P=0.000).

Likewise, in 25, 50, 100 umol/L NaHS group, the ATP level was also highly positively correlated with HO-1, MnSOD protein level (r_{HO-1}=0.912, P=0.000; r_{Mn-SOD}=0.915, P=0.000), the ROS level was moderately negatively correlated with HO-1, MnSOD protein level (r_{HO-1}=-0.501, P=0.002; r_{Mn-SOD}=-0.511, P=0.002), the MDA level was highly negatively correlated with HO-1, MnSOD protein level (r_{HO-1}=-0.966, P=0.000; r_{MnSOD}= -0.965, P=0.000).

Discussion

With the use of CLP-induced sepsis, a well-established and clinically relevant animal model for sepsis, our findings indicated that exogenous NaHS could promote the expression of HO-1 and MnSOD, lessen oxidative stress, and improve the myocardial mitochondria function in CLP-induced sepsis.

Mitochondria are the powerhouse of the cells. Along with energy production, mitochondria regulate several physiologic and pathologic

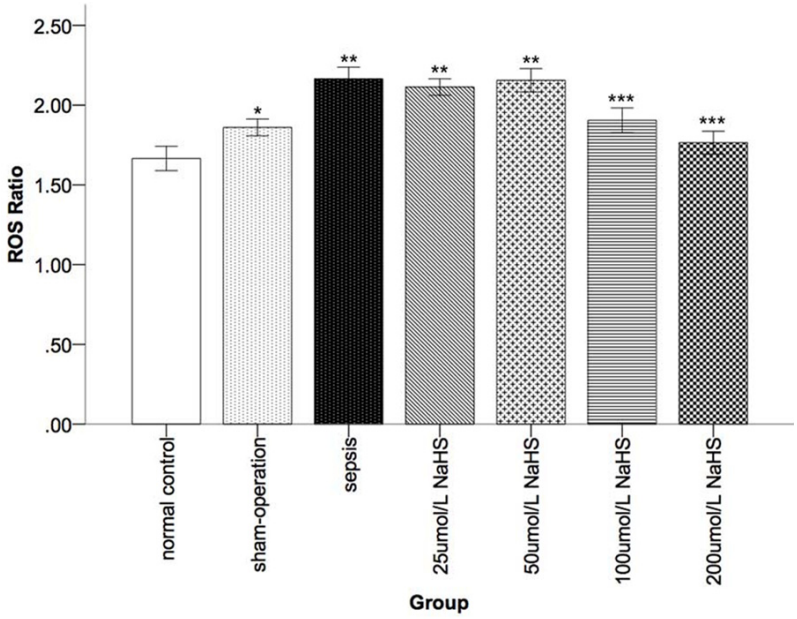

Figure 2. The ROS level of each group (n=10 per group). The mice were submitted to CLP procedure for sepsis induction. Meanwhile, intraperitoneal injection of NaHS in corresponding concentration was administered. 12 h later, the mice were euthanized and myocardial tissue collected for subsequent analysis. The ROS level was detected by spectrofluorometer. *P<0.05 vs. normal control group, **P<0.05 vs. sham-operation group, ***P<0.05 vs. sepsis group. The error bars are indicative of the 95% confidence intervals.

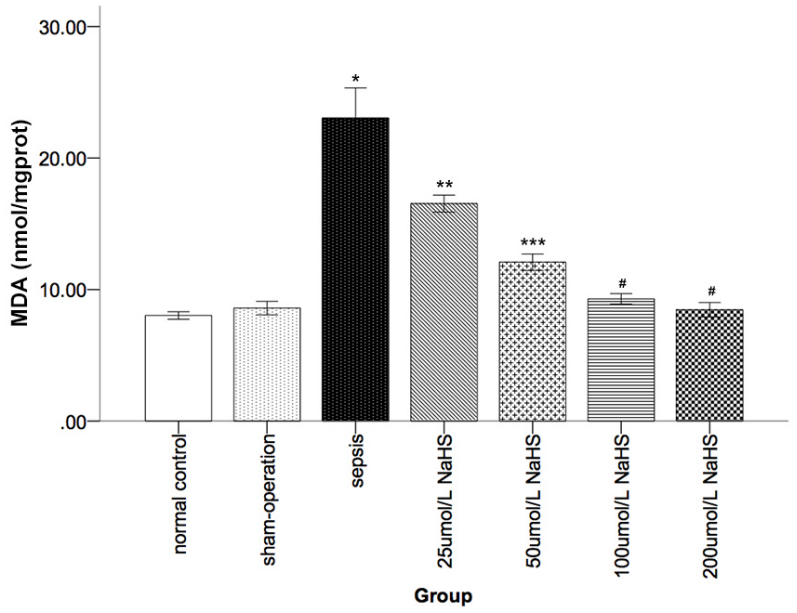

Figure 3. The level of MDA in each group (n=10 per group). The mice were submitted to CLP procedure for sepsis induction. Meanwhile, intraperitoneal injection of NaHS in corresponding concentration was administered. 12 h later, the mice were euthanized and myocardial tissue collected for subsequent analysis. The MDA level was detected by TBA method.*P<0.05 vs. sham-operation group, **P<0.05 vs. sepsis group, ***P<0.05 vs. 25 umol/L NaHS group. #P<0.05 vs. 50 umol/L NaHS group. The error bars are indicative of the 95% confidence intervals.

reactions, including apoptosis, ROS generation, and detoxication [14]. Mitochondrial abnormalities in sepsis-both ultrastructural and biochemical-have been reported as long as 30 years ago [15]. However, the effects of sepsis on mitochondrial function remained controversial. There is considerable variability among the results of the studies in the literature, with reports of increased, decreased, or unaltered mitochondrial function in sepsis [16-18]. This study indicated that the myocardial mitochondria had obvious swelling in the sepsis group. The ATP level of the sepsis group was decreased significantly. All these results supported that the myocardial mitochondria were damaged in sepsis.

During sepsis, a biochemical mechanism within the mitochondria where the oxidation of succinate to fumarate by succinate dehydrogenase is reversed, which leads to succinate accumulation. Then, oxygen administration (e.g. reperfusion) rapidly oxidizes the accumulated succinate, leading to the generation of large amounts of superoxide radical and other free radical species [19]. The result of this study indicated that the ROS ratio, as well as the level of MDA, increased significantly in sepsis, which showed that myocardial mitochondria might be damaged by oxidative stress during sepsis. These were consistent with previous studies [20, 21]. Being the major source

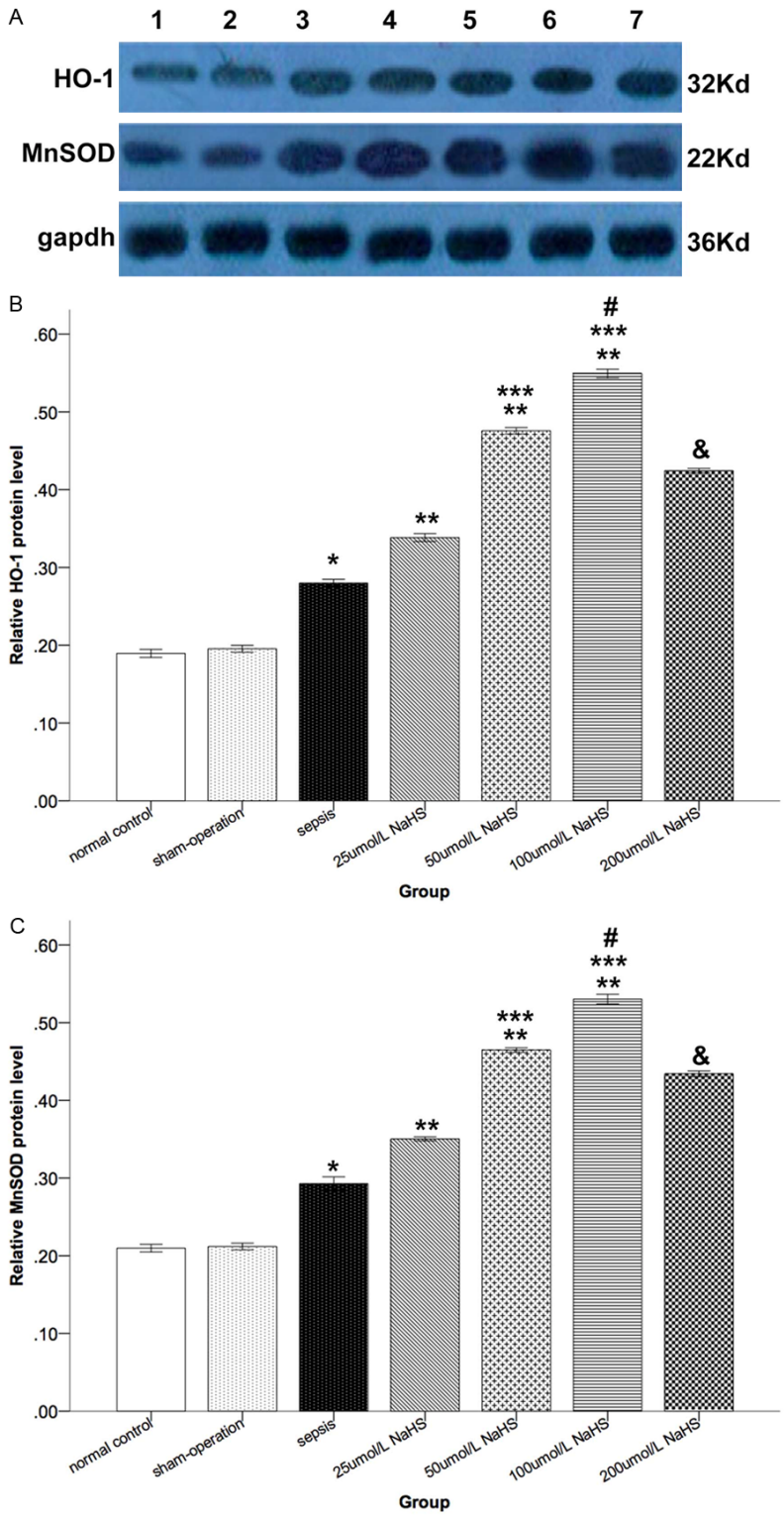

Figure 4. (A) Western blotting analysis of the protein expression level of HO-1 and MnSOD. (B, C) The bands obtained from (A) were scanned for densitometry analysis and compared between each group. *P<0.05 vs. sham-operation group, **P<0.05 vs. sepsis group, ***P<0.05 vs. 25 umol/L NaHS group, #P<0.05 vs. 50 umol/L NaHS group. &P<0.05 vs. 100 umol/L NaHS group. The error bars are indicative of the 95% confidence intervals.

of intracellular ROS, mitochondria are a major target of ROS-mediated damage [22]. Mitochondrial ROS are tightly regulated by endogenous antioxidant scavenging systems. The antioxidant defense systems are tightly regulated and consist of a combination of enzyme and non-enzyme pathways. HO-1 and MnSOD are representative antioxidant enzymes that play important roles for ROS detoxification [23, 24]. It has been found that the reduction of antioxidant defense systems are associated with mitochondrial dysfunction and play important roles in the pathogenesis of sepsis [16, 25]. It was suggested that these observations are most likely a result of proteolysis of the heparin-binding domain rather than alterations in the expression of antioxidant enzymes [26, 27]. However, we found that the mRNA and protein expression level of HO-1 and MnSOD in the sepsis group increased significantly. It may be induced by inflammatory factors during sepsis, such as IL-10 [28, 29], but this is not sufficient to prevent oxidative damage. Swelling and dysfunction of myocardial mitochondria also were observed in this study.

Since mitochondrial damage caused by oxidative stress is the main reason for myocardial injury in sepsis, antioxidant therapy might be an effective treatment for sepsis. H_2S has been shown to have protective effects by down regu-

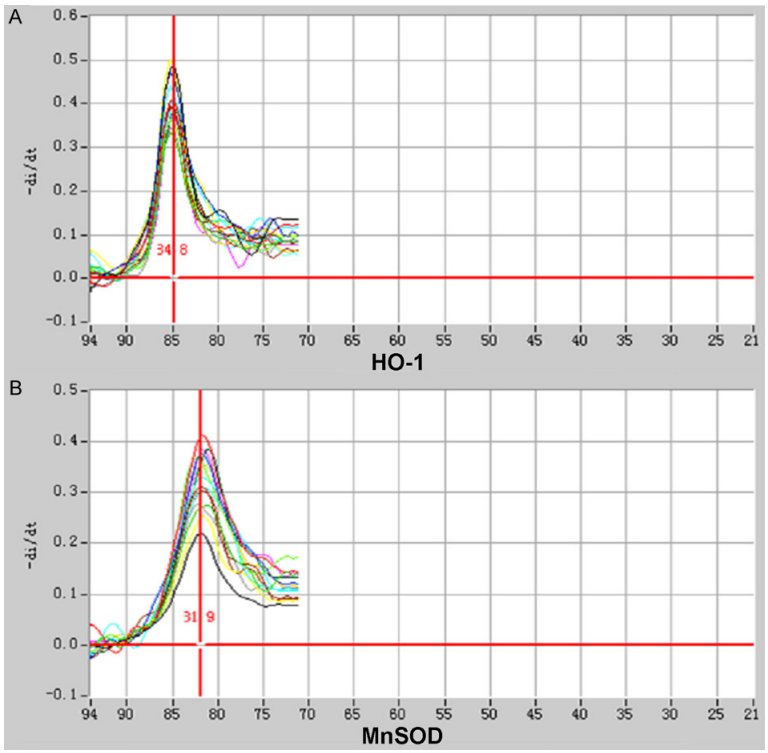

Figure 5. Melting curve of HO-1 and MnSOD.

lating oxidative stress in models of ischemia/reperfusion [30], smoke inhalation injury [31], allergy [32], and by inhibiting C/EBP homologous protein 10 (CHOP), improving neutrophil migration in sepsis [33, 34]. But few studies regarding effects of H_2S on oxidative stress induced by sepsis. In this study, we found that the degree of myocardial mitochondria swelling was alleviated and the ATP level was increased after NaHS treatment, in a concentration-dependent manner. It displayed that exogenous NaHS, as a donor of H2S, had a concentration-dependent protective effect on myocardial mitochondrial damage induced by sepsis. In addition, we also demonstrated that NaHS treatment could not only reduce the ROS and MDA level but also improve the mRNA and protein expression level of HO-1 and MnSOD concentration-dependently. Moreover, in NaHS treatment group, the ATP level was positively correlated with HO-1, MnSOD mRNA level, and the ROS, MDA level were negatively correlated with HO-1, MnSOD mRNA level. The similar correlation were found between the ATP, ROS, MDA level and HO-1, MnSOD protein level in 25, 50, 100 umol/L NaHS treatment group. This suggested that NaHS could promote the expression level of HO-1, MnSOD and lessen oxidative stress, which were correlated. In addition, we found that NaHS inhibited oxidative stress in a concentration-dependent manner. The inhibiting effect strengthened as the NaHS concentration increased. But the protein level of HO-1 and MnSOD in the 200 umol/L NaHS group were lower than that in 100 umol/L NaHS group. That might be due to the inhibition effect on post-translational modification of protein of NaHS at high concentration [35]. Therefore, the concentration of NaHS at 100 umol/L as most suitable for the experiment.

In conclusion, this study demonstrated that myocardial mitochondria were damaged by oxidative stress in CLP-induced sepsis. Exogenous NaHS had a concentration-dependent protective effect on myocardial mitochondria in sepsis. Exogenous NaHS also could promote the expression of HO-1 and MnSOD, and lessen oxidative stress. This might be the mechanism of NaHS protecting myocardial mitochondria, which should be studied further on cell lines by blocking the elevation of HO-1 and MnSOD. In addition, exogenous NaHS may be an effective treatment for sepsis, but the protective availability and therapeutic concentration of NaHS should facilitate further studies in patient-specific studies.

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Disclosure of conflict of interest

None.

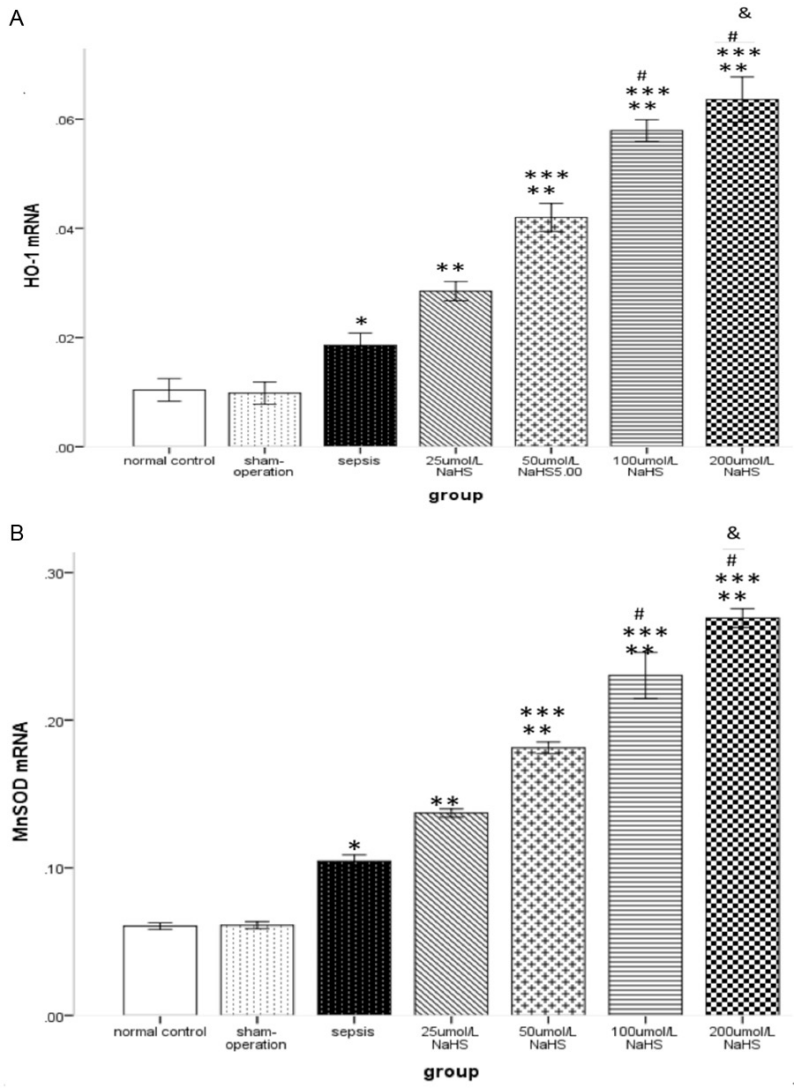

Figure 6. The relative quantification compared between each group. *P<0.05 vs. sham-operation group, **P<0.05 vs. sepsis group, ***P<0.05 vs. 25 umol/L NaHS group, #P<0.05 vs. 50 umol/L NaHS group. &P<0.05 vs. 100 umol/L NaHS group. The error bars are indicative of the 95% confidence intervals.

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